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14. ABSTRACT: The c-Myc oncogene is amplified and/or overexpressed in a large number of human malignancies including a significant proportion of breast cancers. It encodes a potent transcription factor that binds to E-box present in promoter regions of many cancer-relevant genes. Despite its prominent role in transformation and cancer, we know very little about its role in breast cancer progression. To understand its role in breast cancer and human mammary (or breast) epithelial cell (HMEC) transformation, we overexpressed c-Myc in HMECs. In this report, we show that overexpression of c-Myc can transform HMECs. We also report that c-Myc overexpression leads to upregulation of polycomb group proteins BMI1 and EZH2. Upregulation of PcG proteins by c-Myc was accompanied by increased histone posttranslational modification activities of PcG proteins. In particular, c-Myc increased the levels of H3K27Me3 (mediated by EZH2). We also report that Myc overexpression led to downregulation of Wnt inhibitors DKK1 and SFRP4, and upregulation of Wnt family members such as WNT2, WNT3, WNT4, WNT5B, WNT7A and WNT16. Since PcG proteins also inhibit expression of Wnt inhibitors, our data suggest that the downregulation of Wnt inhibitors and upregulation of Wnt family members may be mediated by PcG proteins BMI1 and EZH2 that are induced by c-Myc. In summary, Myc, PcGs, Wnt inhibitors and Wnts act in a linear pathway to transform breast epithelial cells.					
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Introduction:

c-Myc is amplified and/or overexpressed in a large number of human malignancies including breast cancer [1]. In addition, various *Myc* transgenic mice and xenograft models strongly implicate it in the etiology of breast cancer. Myc encodes a potent transcription factor that binds to E-box present in promoter regions of many cancer-relevant genes. It can also repress transcription of its target genes independent of its binding to E-box. Despite its prominent role in transformation and cancer, we know very little about its role in breast cancer progression. Recently it was shown that c-Myc alone can transform human mammary epithelial cells (HMECs) in culture [2]. Increased Wnt signaling has also been shown to transform primary HMECs [3]. Recent work from several laboratories suggests a prominent role of PcG proteins and Wnt signaling in breast cancer [2, 4], and link Myc to regulation of PcG proteins, in particular Bmi-1 [5, 6]. PcG proteins function as transcriptional repressor and are involved in gene silencing. These proteins repress many differentiation-specific genes and tumor suppressors including Wnt inhibitors. In this proposal, we tested a hypothesis that Myc transforms HMECs via upregulation of polycomb proteins BMI1 and EZH2, which repress expression of Wnt inhibitors resulting in activation of Wnt family members.

Body: The approved statement of work included following tasks/aims-

Task 1- Develop an inducible c-Myc based transformation model of HMECs and determine if induction of c-Myc leads to upregulation of BMI1 and EZH2. Task 2- Determine if induction of BMI1 and EZH2 by c-Myc leads to increased histone posttranslational modifications (PTMs) associated with expression of PcG proteins and polycomb repressive complexes (PRCs). Task 3- Determine if BMI1 and EZH2 upregulation and histone PTMs associated with them leads to downregulation of Wnt inhibitors. Task 4- Determine if downregulation of BMI1 and EZH2 impacts expression of Wnt inhibitors and c-Myc-induced transformation of breast epithelial cells in culture. We completed following tasks/aims during this funding period (1 year)-

1. We developed an inducible c-Myc based transformation model of HMECs and determined that the induction of c-Myc led to upregulation of BMI1 and EZH2. 2. We determined that the induction of c-Myc leads to increased histone PTMs associated with expression of PcG proteins and polycomb repressive complexes (PRCs). 3. We determined the effect of Myc induction on regulation of Wnt inhibitors and Wnt family members. Due to time constraints, we were not able to complete the task 4. The results are summarized as follows-

1. c-Myc inducible HMEC lines: We have recently reported that c-Myc is a positive regulator of BMI1 in human fibroblasts and that it induces BMI1 [6]. In addition, we analyzed 5' region of EZH2 for potential E-box sequences to which MYC-family of transcription factors can bind. Our analysis of ~2.0 Kb of *EZH2* promoter indicated that indeed *EZH2* promoter contains a perfect E-Box (GACACGTGCT) at -1103 bp in its upstream region. Thus it is likely that c-Myc induces both BMI1 and EZH2. To demonstrate that c-Myc indeed regulate EZH2 and BMI1 expression, we derived stable 76NhTERT (telomerase immortalized normal HMECs) and MCF10A (spontaneously immortalized non-transformed cells) cell lines, expressing a 4- Hydroxy-tamoxifen (4-OHT) inducible c-Myc (kindly provided by Dr. G. I. Evan, UCSF, San Francisco) [7]. To develop these cell lines, 76NhTERT and MCF10A cells were infected with a retroviral vector expressing OHT-inducible Myc-ER, and selected in puromycin. Control cell lines infected with vector alone (B0) were also selected in puromycin.

2. c-Myc induction leads to upregulation of BMI1 and EZH2 in HMECs: After stable selection, cell lines were studied for inducible c-Myc expression using -OHT and +OHT culture conditions (Fig.1A), and to determine whether OHT-mediated induction of c-Myc leads to overexpression of PcG proteins BMI1 and EZH2. Overexpression of PcG proteins was studied using western

blot analyses. Results indicated that induction of c-Myc by OHT led to dose-dependent upregulation of BMI1 and EZH2 (Fig. 1A). Next we determined whether similar to BMI1 [6], Myc transcriptionally upregulates EZH2. Transcriptional upregulation of EZH2 by Myc was studied by RT-PCR (reverse transcription-polymerase chain reaction). Results indicate that indeed EZH2 is upregulated by c-Myc induction at the mRNA levels (Fig. 1B) suggesting transcriptional regulation of EZH2 by c-Myc.

3. c-Myc induction leads to transformed phenotype in HMECs- To determine whether induction of c-Myc by OHT in this model is sufficient to transform immortalized HMECs, we performed Matrigel and soft agar assays. The assays were performed as described [4, 8]. Results indicated that c-Myc induction leads to transformation of immortalized HMECs.

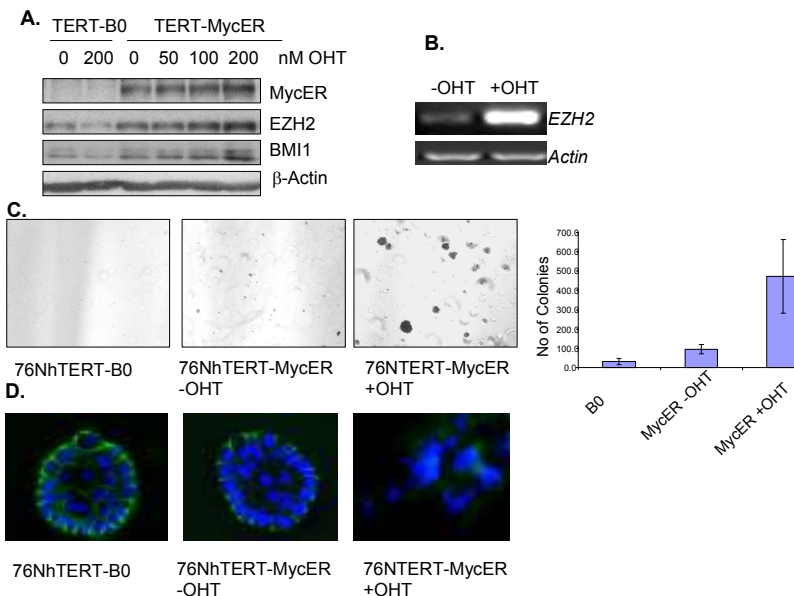


Figure 1: c-Myc-overexpression leads to induction of EZH2 and BMI and transformation of HMECs. **A.** 4-OHT-inducible c-Myc (MycER) was introduced into 76NhTERT cells using pBabe-MycER retrovirus. Control B0 and c-MycER cells were first grown in phenol-red free HMEC growth medium and then treated with different concentrations of 4-OHT (0-200 nM) for 16 hrs. Total cell lysates were prepared and western blot analysis for c-Myc, BMI1 and EZH2 was performed. Results indicated a dose-dependent increase in expression of BMI1 and EZH2 upon c-Myc induction by 4-OHT. **B.** To determine if EZH2 is also a direct transcriptional target of c-Myc, we performed RT-PCR analysis of 76NhTERT-MycER cells with and without 4-OHT treatment. Results indicated a strong transcriptional induction of EZH2 by MycER within 1 hr of OHT-mediated induction of c-Myc suggesting that EZH2 is a direct transcriptional target of c-Myc. **C and D.** 4-OHT-induction of c-Myc also led to transformation of 76NhTERT cells as determined by soft-agar growth and Matrigel assays respectively. Exogenous c-Myc was induced in 76NhTERT-MycER cells using different concentrations of 4-OHT (as indicated). Western blot, RT-PCR, soft agar growth and Matrigel analyses were performed as described [48, 51-53]. Results of soft agar and Matrigel assays indicated that upon c-Myc induction, these cells are able to grow in anchorage-independent manner in soft agar and make irregular or no acini, which is indicative of transformation of these HMEC by c-Myc. As expected control cells did not grow in soft agar and make regular acini in Matrigel. c-Myc induction also leads to loss of E-cadherin indicative of epithelial to mesenchymal transition (EMT). Note that MycER contains a background leaky c-Myc activity, which explains partial induction of EZH2 and BMI1, few colonies in soft agar and some loss of acinar morphology even without OHT.

4. c-Myc induction leads to upregulation of histone PTMs associated with PcG protein EZH2. EZH2 is a constituent of PRC2 (polycomb repressive complex 2), which possesses histone methyl transferase activity. It specifically trimethylates Lys 27 residue of Histone 3 (H3K27Me3), which is associated with gene silencing. To determine whether upregulation of EZH2 by c-Myc results in increased H3K27Me3 levels, we examined the levels of H3K27Me3 in control MCF10A and MCF10A-MycER cells under -OHT and +OHT conditions by immunostaining using a rabbit polyclonal antibody specific to H3K27Me3 (Fig. 2B).

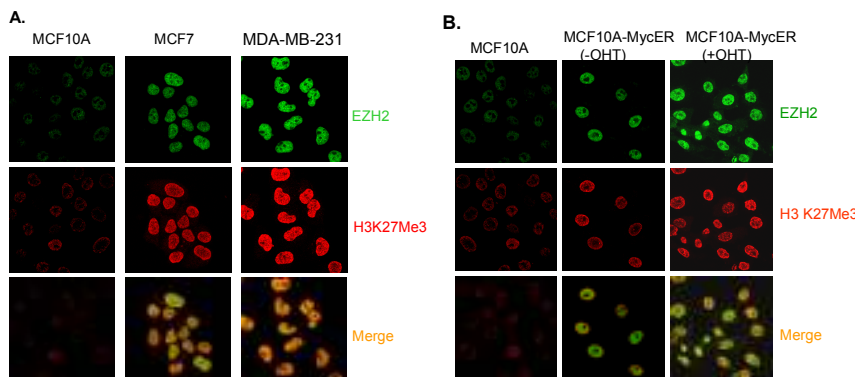


Figure 2: **A.** MDA-MB-231 cells express high EZH2 and correspondingly high H3K27Me3 activity compared to MCF10A cells. MCF7 cells contain intermediate levels of EZH2 and H3K27Me3 activity. **B.** c-Myc upregulates H3 K27Me3 activity. c-Myc induction by 4-OHT in MCF10A-MycER, was achieved using 200 nM 4-OHT as described in Figure 1. Indicated cells were immunostained using a mAb against EZH2 (BD Biosciences) and a rabbit polyclonal against H3K27Me3 (Cell Signaling).

5. c-Myc induction results in differential regulation of Wnt signaling molecules- To determine whether c-Myc impacts Wnt pathway by regulating Wnt inhibitors and Wnt factors, we performed an array analysis of the Human Wnt Signaling Pathway RT² Profiler PCR Array (SABiosciences)

The array contains 84 genes related to Wnt pathway-

(http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-043A.html). The mRNA was prepared from control MCF10A B0 cells (+OHT) and MCF10A-MycER cell (+OHT) condition to avoid the leaky background expression of c-Myc and to minimize the potential contribution of OHT on other genes in HMECs. The array analysis was performed as described by the supplier. Our results indicated that as expected MCF10A-MycER (+OHT) cells had upregulation of c-Myc compared to B0 cells. MycER cells also upregulated various WNT factors. The results also demonstrated downregulation of two important Wnt inhibitors DKK1 and SFRP4. The data are summarized in following table-

Upregulated Genes		Downregulated Genes	
Gene	Fold Change	Gene	Fold Change
MYC	3.2	DKK1	-2.3
BCL9	2.1	SFRP4	-4.0
WNT2	1.4	SFRP1	-1.3
WNT3	1.6	SENP2	-6.3
WNT4	1.4	GSK3B	-1.4
WNT5A	1.7	FOSL1	-2.0
WNT5B	4.6	PYGO1	-2.7
WNT7A	2.2	LEF1	-2.6
WNT16	4.9	FZD3	-1.6

Thus, these data suggest that Myc upregulates Wnt pathway possibly by downregulating DKK1, SFRP1 and SFRP4. Myc may also directly upregulate various WNT family members. The detailed regulation of various Wnt pathway genes identified in this experiment remained to be studied and confirmed.

Key Research Accomplishments:

1. We developed c-Myc inducible HMEC lines.
2. We demonstrated that Myc upregulates BMI1 and EZH2 in HMECs.
3. Myc upregulates H3K27Me3 activity of PRC1.
4. Inducible Myc is sufficient to transform HMECs.
5. Myc inhibits several Wnt inhibitory factors possibly via upregulation of BMI1 and EZH2.
6. Myc upregulates certain Wnt family members.

Reportable Outcomes: This concept grant partially supported studies presented in following publications-

1. Dimri, G.P. (2009) c-Myc and Telomerase activation. *Cell Cycle* 8: 3075-3076.
2. Bommi, P., Dimri, M., Sahasrabudhi, A.A., Khandekar, J.D., and G.P. Dimri (2010) The polycomb group protein BMI1 is a transcriptional target of HDAC inhibitors. *Cell Cycle*, July 21; 9(13) [Epub ahead of print].

3. Yadav, A.K., Sahasrabudde, A. A., Dimri, M., Bommi, P.V., Sainger, R. and G. P. Dimri (2010) Deletion analysis of BMI1 oncoprotein identifies its negative regulatory domain. *Mol Cancer*. 2010 Jun 22;9:158.

Conclusion: From our studies presented here which were supported by this concept grant, we concluded that Myc is a positive transcriptional regulator of EZH2 in addition to BMI1. Although, our studies also showed that Myc can downregulate Wnt inhibitors, it is not yet clear whether BMI1 and EZH2 are directly involved in repression of Wnt inhibitors. Further studies are required to establish a direct link between Myc, PcG proteins, Wnt inhibitors and Wnt factors-HMEC transformation.

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Appendices: None